Macrothink Institute™

Journal of Biology and Life Science
ISSN 2157-6076
2024, Vol. 15, No. 1

Assessment of Proteinase and Phospholipases Enzymes Isolated from Pathogenic *Candida* species from Women Attending Antenatal Clinic at Mbeya Zonal Referral Hospital, Southern Highland Regions of Tanzania

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Received: October 27, 2023 Accepted: January 26, 2024 Published: February 17, 2024

doi:10.5296/jbls.v15i1.21717 URL: https://doi.org/10.5296/jbls.v15i1.21717

Abstract

Fungi are a global cause of vaginal infections, with vaginal candidiasis largely afflicting tropical regions including most parts of sub-Saharan Africa. In Tanzania, baseline studies have established the prevalence of *Candida* species among symptomatic and asymptomatic pregnant women in Dar es Salaam (Namkinga *et al.*, 2005; Namkinga 2012) and in Mwanza (Mushi *et al.*, 2019). However, no study that has been done to determine the above-mentioned enzymes form *Candida* species in Southern Highland Regions of Tanzania. Several factors have been reported to contribute to the virulence and pathogenicity of *Candida*, among others the production of extracellular hydrolytic enzymes, particularly phospholipase and proteinase. Therefore, this study was designed to investigate the *in vitro* production of phospholipases and proteinases enzymes from isolated pathogenic *Candida* spp from pregnant women.

Of the 280 samples collected from symptomatic and asymptomatic pregnant women attending antenatal clinics at Mbeya Zonal Referral Hospital were tested for several microbiological methods such as the wet mount microscopic preparations (hanging drop) and the Gram stained smears for microscopy, cultures on Sabouraud's dextrose agar and on Conidia enhancing medium (Corn meal agar), germ tube test, biochemical tests, sugar fermentation-assimilation tests and molecular test to characterize *Candida* species.

Phospholipase production was performed in egg yolk medium while the production of proteinase was done in a medium containing bovine serum albumin. All analyses were performed in triplicates. The results showed that; out of the 280 tested, 155 (74.3%) isolates were phospholipase positive while 201 (96.6%) were positive for proteinase activity. *C.*

albicans was the species with the highest number of positive isolates for proteinase and phospholipase 110 (95.6%), and most strains of *C. albicans* produce both enzymes (phospholipases and proteinases). The non-albicans *Candida* isolates were also producers of hydrolytic enzymes that, consequently, might be able to cause infections as favorable conditions arises.

Keywords: *Candida*, antenatal clinic, hydrolytic enzymes, virulence factors

1. Introduction

The genus *Candida* especially *Candida albican*s, are known to be the normal body flora in buccal cavity and women's genitalia. More than 90% of invasive infections caused by *Candida* species have been linked merely to few spp.; *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei* among all *Candida* (Papon *et al*., 2013), where 70% out of which are associated to *C. albicans* (Benito, *et al*., 2005, Al-Musawi *et al*., 2021) being habitant of various location of human body like oral cavity, digestive tract, vagina and skin, species of *Candida* constitute normal flora microbiome's components (Seneviratne, *et al*., 2008) with opportunistic pathogenicity mostly occur in cases of immune-compromised states of individuals health. Because of host debilitation or change in the local environment that promote *Candida* growth, opportunistic infections by such normal flora become viable (Gow *et al.*, 2011) and therefore resulting into symptomatic candidiasis like mucosal and cutaneous candidiasis as well as systemic candidiasis. Clinical incidences of candidiasis have been increasing in due to various factors which lead to compromised immunity such as HIV/AIDS pandemic, increased use of antibiotics, organ transplantation and the use of invasive devises (Rokas, 2022). The transit from a harmless commensal to disease causing pathogens and vice versa by *Candida* is based on the compromise immune system of the host relative to *Candida* virulence (Yang, 2003).

The virulence factors that contribute to the transformation from harmless commensals to pathogenic forms under the conditions of a dysfunctional host defense system include adhesions proteins, germ tube and mycelia formation as well as secreted proteolytic and hydrolytic enzymes. Indicators of *Candida* virulence have therefore been their ability to adhere to host tissue and secretion of those virulent extracellular enzymes which promotes both dimorphic transition (Colombo *et al.*, 2003) and penetration of *Candida* to host cells. Adherence to the host tissue, which pave to colonization and subsequent infection, is considered as an early stage of infection by *Candida* (Haynes 2001). Secreted virulent enzymes, mainly proteinases and phospholipases, are believed to play an important role in both *Candida* overgrowth as they facilitate adherence as well as their penetration into host tissue (Tsang *et al.*, 2007). Proteinase is an aspartyl acid protein enzyme secreted by *C. albican, C. parapsilosis,* and *C. glabrata*. This enzyme causes limited proteolysis of Hageman factor, leading to activation of the Kallikrein-kinin system, which in turn generates bradykinin and cause increased vascular permeability and inflammation (esp. *C. albicans, C. parapsilosis, C. glabrata*) (Hube, 1996, Watts, *et al*., 1998, De Bernardis, *et al*., 1989, De Bernardis, *et al*., 1999).

Proteinase may also aid invasion through the keratin protective layer & facilitate initiation of

cutaneous candidiasis (Negi, *et al*, 1984, Hube & Naglik, 2001)

Women with candida vaginitis from *Candida* spp. Secreting proteinase enzymes will have severe vulvo-vaginal itching and inflammation, due to histamine bradykinin.

Phospholipases secretion; these extra-cellular membrane damaging enzymes are considered to be virulence factors for *C. albicans* and *C. glabrata.* Some of these enzymes are capable of hydrolyzing even the ester bonds in glycerides (Naglik *et al*., 2003, Barret-Bee, *et al*., 1985, Ibrahim, *et al*., 1995). Recently there have been numerous reports on differences in virulence among the genotypes distinct *Candida* spp (de Azevedo Izidoro *et al.,* 2012), which correlates with expressions of virulence factors. This study aimed to investigate the *in vitro* activity of phospholipases and proteinases enzymes in clinical isolates of *Candida* spp. Moreover, the study characterized *Candida* spp. in order to associate species of Candida and the enzyme identified (Kantarcioglu and Yücel, 2002).

2. Materials and Methods

Collection of High Vaginal Swabs Samples according to Namkinga (2012)

Total of 280 high vaginal swab (HVSs) samples were collected from both symptomatic and asymptomatic pregnant women attended antenatal clinic at Mbeya Zone Referral Hospital (MZRH) between January 2020 and June, 2021. All participated women, aged 15 to 45 years granted their consent to provide samples for this study. High vaginal swabs were collected using sterile cotton swabs aseptically. All collected samples were kept aseptically to laboratory (Department of Microbiology and Immunology, UDSM-MCHAS). In the laboratory, samples were analyzed microscopically as wet mount to know the presence of yeast cells. Then the samples were stored at 4^{o}C for further analysis.

Morphological Identification

Swab samples were subjected for examination by staining and observed microscopically and were also cultured on Sabouraud's dextrose agar (SDA) (Oxoid Ltd. Hampshire, England), supplemented with 0.005% chloramphenicol and 0.05% cycloheximide and incubated at 25 ºC. Samples were analyzed microscopically after gram staining then were re-analyzed again after every 24 hrs, if no visible growth samples were re-incubated to a maximum of 72 hrs before discarding.

Sub-culture

Sub-culturing was done to obtain pure cultures. The pure cultures were isolated on SDA medium and incubated at 25^{o}C. The isolates were analyzed microscopically to see the uniformity of yeast growth. The plates containing pure cultures were wrapped with parafilm to avoid contamination then stored in refrigerator at 4 ºC for further analysis.

Germ tube test

A loopful of yeast colony from stored pure culture was emulsified into a 0.5 ml of human serum in a small tube and incubated into water bath at 37 ºC for 3 hrs first and analyzed microscopically. A drop of the incubated serum was placed on a microscopic glass slide,

covered by cover-slip and examined by the microscope for the presence of sprouting cells (germ tubes) after every one hour for the identification of *C. albicans.* The incubation was continued for 24 hrs more in case of non albicans germ tube producers.

Enzyme Activities

Before evaluation of hydrolytic enzymes activities, the yeasts Candida were subjected to characterization phenotypically and genotypically.

Phenotypic characterization was done by; culture on Sabouraud's dextrose agar, culture on corn meal agar containing tween 80 and production of germ tube in human serum.

Genotypic characterization - Species identification was done by PCR method and genetic relatedness was assessed by randomly amplified polymorphic DNA analysis (RAPD) using ten primers (Namkinga, 2012, Kanishka, *et al.,* 2019).

3. Results

Germ Tube Formation Test

C. albicans isolates showed positive results that the formation of germ tubes was observed within the first 3 hrs of incubation in the water bath. A total of 182 out of 280 (65%) showed positive germ tube results. RAPD test revealed; *C. albicans* n=115, *C. glabrata* n=51, *C. parapsilosis* n=5, *C. tropicalis* n=20, and *C. krusei* n=89 and other non-*Candida* yeasts cells which were neglected during evaluation of enzymes activities.

Phospholipase activities

The ability of *Candida* spp to produce phospholipases was determined on egg-yolk plates. Ten microliters of saline suspension of *Candida* were inoculated in the wells on egg-yolk plates and incubated at 37^{o}C for 3-7 days. Measurement and calculation of the zone of phospholipase activity (Pz) was done according to the method by (Price *et al*., 1982, Oksuz, *et al.,* 2007).

Proteinase

Proteinase production was verified in agar plates containing bovine serum albumin.

A loop of cells of *Candida* grown on SDA were suspended on saline before inoculated on bovine serum albumin agar and incubated at 37^{o}C for 7 days and the reading was done after every 24 hrs. The formations of a clear zone around colonies (precipitation), which imply the secretion of proteinase by *Candida*, were recorded. (Price, *et al.,* 1982, Oksuz, *et al*., 2007).

Table 1. Positive and negative extracellular enzyme productions by *Candida albicans* and non-*albicans* spp.

Species	Sample size (n)	Phospholipase			Proteinase		
		Positive	Negative	% positive	Positive	Negative	% positive
C. albicans	115	110	4	95.65	110	5	95.65
C. grabrata	51	40	11	78.43	36	15	70.59
C. parapsilosis	5	3	2	60	2	3	40
C. tropicalis	20	15	5	75	14	6	70
C. krusei	89	40	49	49.94	39	50	43.82
Total	**280**	**208**	**71**		**201**	**79**	

Table 2. Distribution of Pz values among *Candida* isolates

Pz value	*C. albicans (n-110)*		Non - *albicans (n=98)*	
	Phospholipase	Proteinase	Phospholipase	Proteinase
<0.69 ++++	37	5	42	6
0.70-0.79 +++	40	45	14	30
0.80-0.89 ++	28	36	14	35
0.90-0.99 +	9	24	28	20
1.00 -	0	0	0	0
Total	110 (95.6%)	110 (95.6%)	98 (59.4%)	91 (55.2%)

Key: Pz = phospholipase/proteinase activity zone; Pz = 1, negative activity; 0.64 < Pz <0.99, positive; Pz ≤0.64, strongly positive

Positivity of phospholipase production was found in 208 (74.3%) while proteinase positive were found in 201 (71.8%) *Candida* spp. The phospholipase positive isolates included *C. albicans* 110 (95.7%); *C. glabrata* 40 (78.4%); *C. tropicalis* 15 (75%); *C. parapsilosis* 39 (60%) and *C. krusei* 40 (49.9%). While the protease positive isolates included *C. albicans* 110 (95.6%); *C. glabrata* 36 (70.6%); *C. tropicalis* 14 (70%); *C. parapsilosis* 2 (40%) and *C. krusei* 39 (43.8%). More-over the distribution of Pz values among isolated *Candida* species was slightly higher among *C. albicans* at Pz value 0.70-0.79 +++ (45) than the non-C. *albicans* at <0.69 ++++ Pz value (42) in Phospholipase activity (Table 2)

4. Discussion

The current study has proved the case that *C. albicans* is and has been long considered as the most common cause of vulvo-vaginal candidiasis infections outnumbering other non-albicans isolates. Similar results of *C. albicans* as the most common etiologic agent of *Candida* infection in women's vaginal site as reported by (Kennedy and Sobel, 2010). While *C. albicans* is the causative agent of over 90% of Vulvo-vaginal candidiais (VVC) cases, other non-*albicans Candida* (NAC) species have also been identified as etiological agents. In some instances, the prevalence of NAC species is disproportionately high, exceeding 50% (Kennedy

and Sobel, 2010). Of the NAC species, *C. glabrata* is regarded as the second leading cause of VVC (~8% of cases), while *C. krusei*, *C. parapsilosis*, and *C. tropicalis* make up a majority of the remainder (Kennedy and Sobel, 2010; Parazzini *et al*., 2000, Richter *et al.*, 2005). Vaginal symptoms resulting from infection with NAC species are often reported as being milder than those experienced during VVC caused by *C. albicans* (Dan *et al.*, 2002). Furthermore, in comparison to studies performed in European and South American countries, *C. parapsilosis* is considered as the most common cause of *Candida* bloodstream infection. This variation may be attributed to the environmental factors, use of central lines, life supporting devices, and antibiotics usages (Oberoi *et al.*, 2012).

Germ Tube Formation Test

The formation of germ tube within the first 3 hrs differentiates *C. albicans* from non-C. *albicans* species. This is in line with Namkinga, (2012). The formation of germ tube is associated with increased synthesis of protein and ribonucleic acid. Germ Tube solutions contains tryptic soy broth and fetal bovine serum, essential nutrients for protein synthesis. It is lyophilized for stability. Germ tube is one of the virulence factors of *Candida albicans.* This is a rapid test for the presumptive identification of *C. albicans* (Namkinga ~~*et al.,*~~ 2012)

Enzymes activities

Candida secretes various extracellular hydrolytic enzymes, which act as important virulence factors (Tellapragada *et al.*, 2014; Canela *et al.*, 2018, Mroczvnska and Brillowska-Dabrowska, 2021). These hydrolytic enzymes degrade the cellular component of tissues and facilitate their survival, adhesion, invasion, and dissemination. Phospholipase, proteinase, lipase, esterase, hemolysin, etc., are the common hydrolytic enzymes. Phospholipase and proteinase hydrolytic enzymes are considered as the two most common virulence factors as they contribute to the *Candida*-host interaction. Proteinase enzyme degrades the surface protein and disrupts the local immunity, resulting in tissue invasion.

Proteinase is an aspartyl acid protein enzyme**.** This enzyme causes limited proteolysis of Hageman factor, leading to activation of the Kallikrein-kinin system, which in turn generates bradykinin **(histamine)** and cause increased vascular permeability & inflammation (esp. *C. albicans*, *C. parapsilosis* Patients with *Candida* spp. that secretes propeinase, experience severe itching in the genitalia.

Phospholipase enzyme degrades the phospholipid component of the cell membrane, resulting in cell damage and lysis that accentuates its dissemination. In our study, higher phospholipase and proteinase enzyme production was shown by the *C. albicans* strains. Canela *et al.*, 2018 and Mutlu-Sariguzel *et al.*, 2015 have reported similar results of higher enzyme production. Our study findings compared to other non-C. *albicans*, phospholipase and proteinase activity (production) were higher in *C. albicans* strains, whereas *C. parapsilosis* and *C. tropicalis* produced far less SAP and PL activities. Our findings have shown that 208(74.3%) and 201(71.8%) positivity in phospholipase and protease production respectively in the isolated *Candida spp*. The phospholipase positive isolates included *C. albicans* 110(95.7%); *C. glabrata* 40(78.4%); *C. tropicalis* 15(75%); *C. parapsilosis* 39(60%) and *C. krusei*. 40(49.9%)

Whereas, the protease positive isolates included of *C. albicans* 110(95.6%); *C. glabrata* 36(70.6%); *C. tropicalis* 14(70%); *C. parapsilosis* 2(40%) and *C. krusei* 39(43.8%).

Candida spp. are opportunistic pathogens related with the increasing rate of life frightening diseases in women with immunocompromised Lim *et al.,* (2021). Proteinase and phospholipase are well thought-out to play essential roles in the pathogenesis of yeast. The roles of these two enzymes in *Candida* spp. and other fungal yeast species seems to be linked to its putative (Pandey *et al.,* 2018). This study focused on phospholipase and proteinase actions in diverse *Candida* spp. isolated from pregnant women. Diverse *Candida* migration rates have been reported with other research Kantarcioglu and Yücel (2002) reported that the positivity rates for phospholipase and proteinase productions were 62.1% and 78.9% respectively, in samples from patients with invasive candidiasis. The phospholipase producing species were *C. albicans*, C. *kefyr, C. lipolytica* and *C. glabrata*. In the same study, proteinase production was observed in *C. albicans, C. kefyr, C. lipolytica, C. parapsilosis* and *C. tropicalis* isolates. (Samaranayake *et al.,* 1984) reported that 73% of their *C. albicans* isolates showed phospholipase and proteinase production. Ibrahim *et al.,* (1995) establish elevated phospholipase production in *C. albicans* isolates that were found in the bloodstream. In the same study phospholipase and proteinase activities reported contrasting enzyme activities and *Candida* spp. (Fotedar and Hedaithy *et al.,* 2003; Hannula *et al.,* 2000).

Moreover, studies on phospholipase and proteinase activities in yeasts isolated from women SSA are limited as the present study aimed at looking on the phospholipase and proteinase activities in yeast. Our findings have shown that the frequencies of phospholipase and proteinase activities in *C. albicans* isolates to be (95.6%); whereas the frequencies of phospholipase and protease activities in non-*C. albicans* isolates were found to be (59.4%) and (55.2%) respectively. Thus, in the present study, the frequencies of phospholipase and proteinase activities were higher than those reported in other studies of enzyme activities in clinical samples (Claudia Spampinato and Dario Leonard, 2013).

5. Conclusion

Candida albicans is the most common isolated yeast from women attending antenatal clinics in the southern highlands of Tanzania. Enzyme assay also showed *C albicans* to produce the highest number of extracellular hydrolytic enzymes and hence *C. albicans* are reported to be the most virulent compared to *non-albicans* species. The solidification of the findings of this study should be supplemented with more samples spanning at a longer time of study and at different study site.

Recommendations

It is recommended that all clinical *Candida* samples to be analyzed to its molecular and enzymatic levels for a specific treatment in pregnant women. Also it is recommended that speciation among *Candida* samples should be a crucial part of the analysis and treatment procedures.

Acknowledgments

I am delighted to convey my thankfulness to many individuals and institutions whose assistance contribute to the completion of this paper. It is worthy to mention that I am cordially indebted to express my sincerely thanks to;

Dr. Malilo (Md) and Dr. Rebecca Mokeha (MD) from antenatal clinic Mbeya Zonal Referral Hospital (MZRH) for their assistance during the whole period of sample collection, also Mr. Kimati for his assistance during the laboratory activities in preparation of all media for identification of yeast, studying morphology formation of *Candida* species and germ tube formation as the features which differentiate *Candida albicans* from non-*Candida albicans*. Miss Winnie Kimaro from the department of Molecular Biology and Biotechnology for her assistance during the laboratory activities in preparation of all media for enzymes production, and studying the presence and absent of enzyme activities. Mr Shauri & Mr. Charles Kayuki for their assistance during the laboratory activities in preparation of media for enzyme activities, guidance and advice without hesitation throughout laboratory work. My acknowledgements are also extended to the academic and technical staff of Chemistry Department and Department of Molecular Biology and Biotechnology, University of Dar es Salaam for their kind assistance at the time of conducting Laboratory activities. Special thanks to my employer, Makete District Executive Director (DED), for giving me the time from the beginning to the final day of accomplishing of this paper.

Authors Contributions

Dr. Joseph Swilla for his assistance during the laboratory activities in studying morphology formation of Candida species and germ tube formation as the features which differentiate *Candida albicans* from non-*Candida albicans*. Dr. Lucy A. Namkinga for her tireless assistance during the laboratory activities in studying phenotypic and genotypic morphology of *Candida* species, also guidance, criticism and encouragement that empowered me to the accomplishment of this paper. She also was a big financial support from data collection, data analysis, write-up until the last drop of the completion of this paper and publication. Dr. Fredrick E. Lyatuu his guidance, criticism and encouragement that empowered me to the accomplishment this paper.

Funding

Special thanks to my employer, Makete District Executive Director (DED), Dr. Lucy A. Namkinga and Dr. Joseph Swilla for funding this study.

Competing interest

The authors declare there is no any competing interest.

Informed consent

Obtained.

Ethics approval

Ethics approval was obtained from Mbeya Zonal Referral Hospital, Ref. number: SZEC-2439/RA/V-1/26 of 11th July, 2019.

Provenance and peer review

Not commissioned; externally double-blind peer reviewed.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Data sharing statement

No additional data are available.

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